KDA Conference 2017

PolyQ Length-Specific Engineering of a HiPSC Model of SBMA Using CRISPR-Cas9 system

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Goal of the project

To further understand motor neuron (MN)-specific molecular mechanisms underlying SBMA

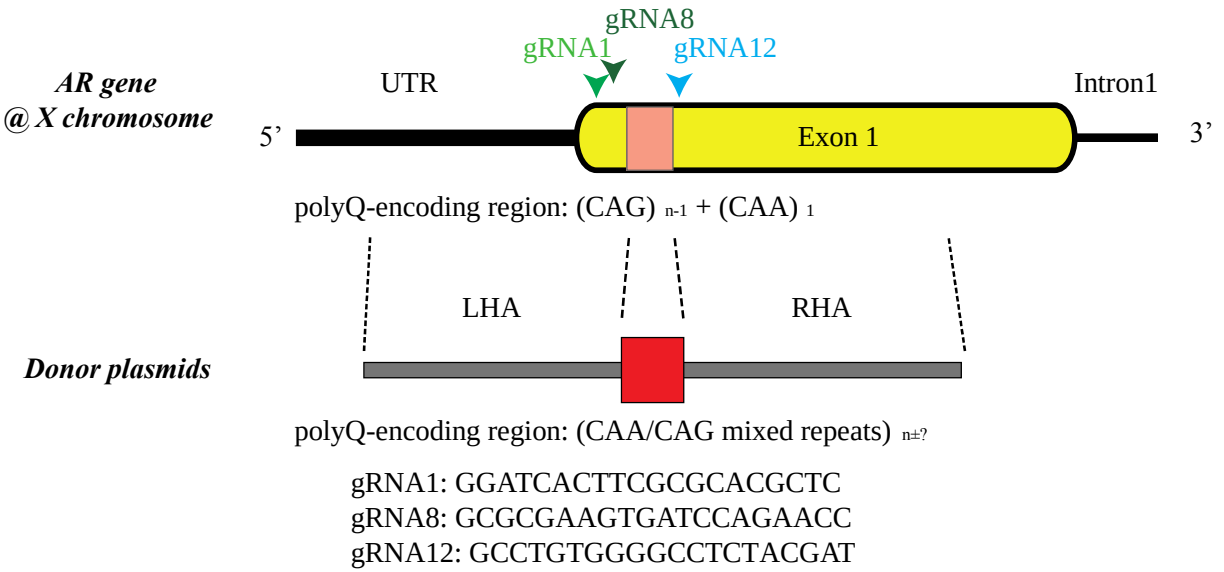
-- by assessing how polyglutamine (polyQ) expansion affects the function and regulation of mutant androgen receptor (AR).

Isogenic human iPSC model of SBMA

- *Human induced pluripotent stem cell (hiPSC) model of SBMA:*
  is a valuable tool for exploring disease mechanisms *(Grunseich et al. 2014)*
  - It is human- and patient-derived
  - It expresses the mutant AR found in a SBMA patient at physiologically appropriate levels
  - hiPSC-derived disease-relevant progenies can be generated in a large quantity in cell culture

- *An isogenic SBMA model:*
  - Possible impacts of genetic background on disease phenotype and variations on expression levels of endogenous AR between individuals.
  - "*Isogenic human disease model:* a family of cells that are selected or engineered to accurately model the genetics of a specific patient population, in vitro" (Wikipedia)
  - Ideally, the only difference across isogenic lines in our SBMA model is the polyQ length in the expressed AR protein.

Engineering using CRISPR-Cas9 system



Schematic of the isogenic engineering using CRISPR-Cas9 system

- *eSpCas9 1.1 endonuclease:* high on-target specificity and robust nuclease activity (*Slaymaker et al. 2016*).
- *gRNAs:*
  - Target 5'-CAG repeat region: gRNA1, gRNA8
  - Target 3'-CAG repeat region: gRNA12
- *Donor:* harbors distinct repeat lengths flanked by long homologous arms

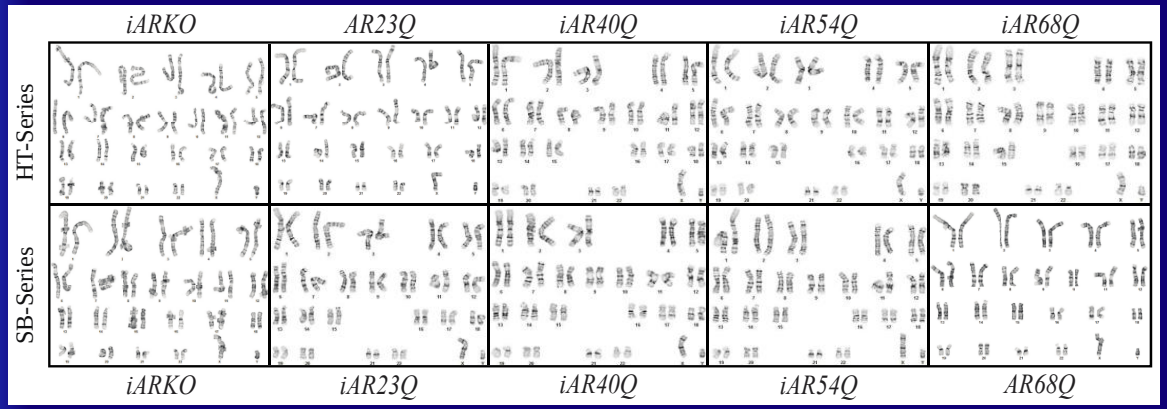
Two series of isogenic lines:

HiPSC series	Cell line name	PolyQ length in AR	Gene knockout/ replacement efficiency (%)
HT-series	*HT-iAR KO*	--	10/16 (62.5%)
	HT-AR23Q	23Q	--
	HT-iAR40Q	40Q	1/46 (2.2%)
	HT-iAR54Q	54Q	2/116 (1.7%)
	HT-iAR68Q	68Q	4/35 (11.4%)
SB-series	*SB-iAR KO*	--	6/16 (37.5%)
	SB-iAR23Q	23Q	3/49 (6.1%)
	SB-iAR40Q	40Q	1/48 (2.1%)
	SB-iAR54Q	54Q	3/78 (3.8%)
	SB-AR68Q	68Q	--

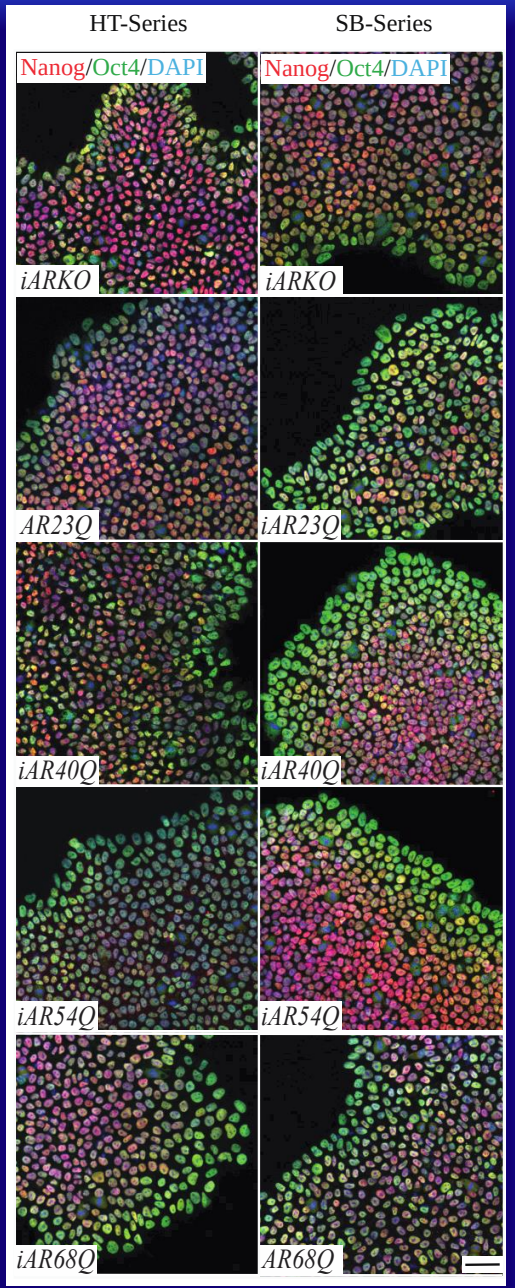
HT-series: derived from a healthy individual expressing AR23Q;

SB-series: derived from a SBMA patient expressing AR68Q

Using CRISPR, gene replacement efficiency ~2-11%; gene knockout efficiency ~37-62%

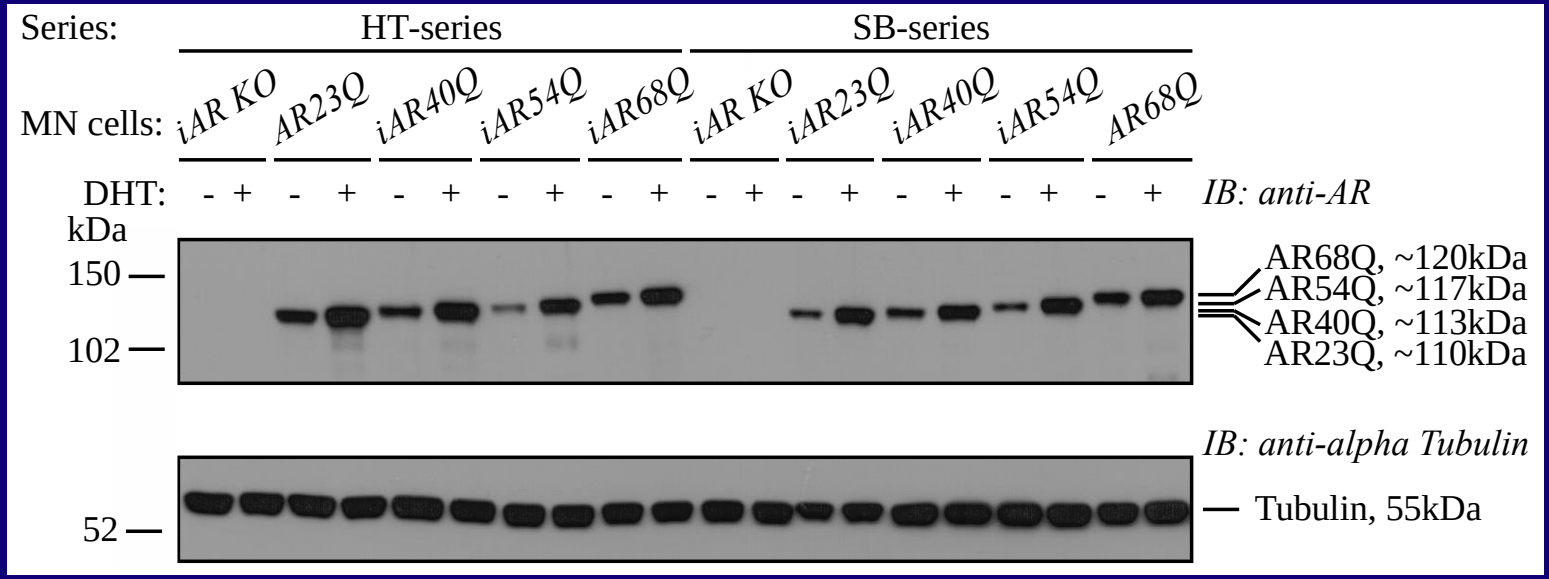


Isogenic lines have normal karyotypes.



Isogenic lines express stem cell markers.

Isogenic lines expressing wildtype or mutant AR



Isogenic lines have compatible AR expression levels and show response to ligand induction.

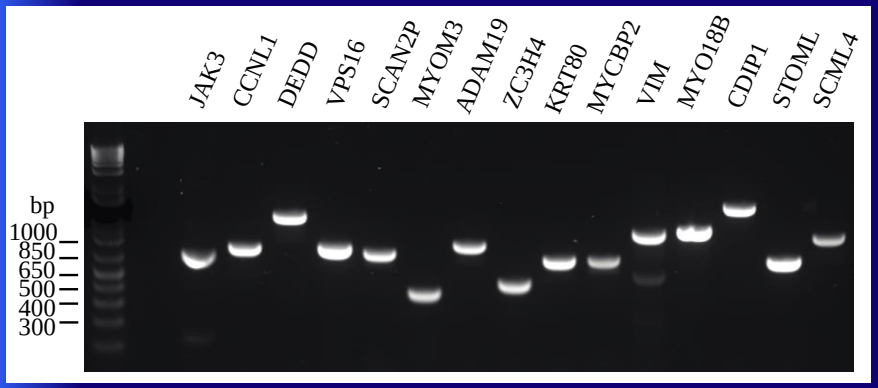
Off-target effect evaluation

Off-target effects:

Sequences in genome similar to target region also potentially can be targeted by gRNAs, leading to erroneous cuts by CRISPR/Cas9 at these "off-target" sites, that introduces mutations. Such unwanted mutations may lead to discrepancies in phenotype.

(Figure source: Harvard news)

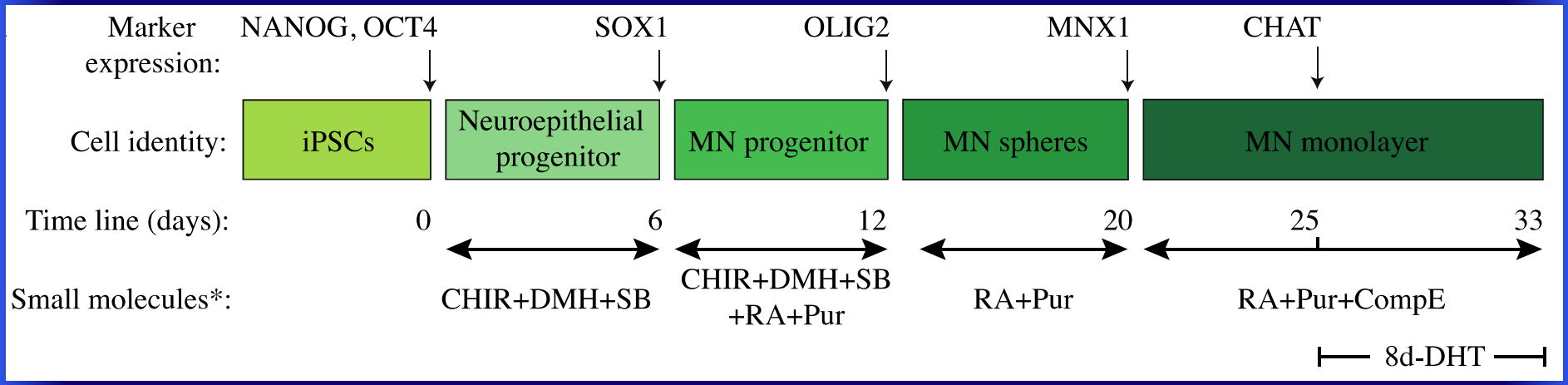
	Gene Name
gRNA1:	**AR**
Off-Targets:	JAK3
gRNA8:	**AR**
Off-Targets:	DEDD
	VPS16
	SCAN2P
	MYOM3
	CCNL1
	ADAM19
	ZC3H4
	KRT80
	MYCBP2
gRNA12:	**AR**
Off-Targets:	VIM
	MYO18B
	CDIP1
	STOML1
	SCML4



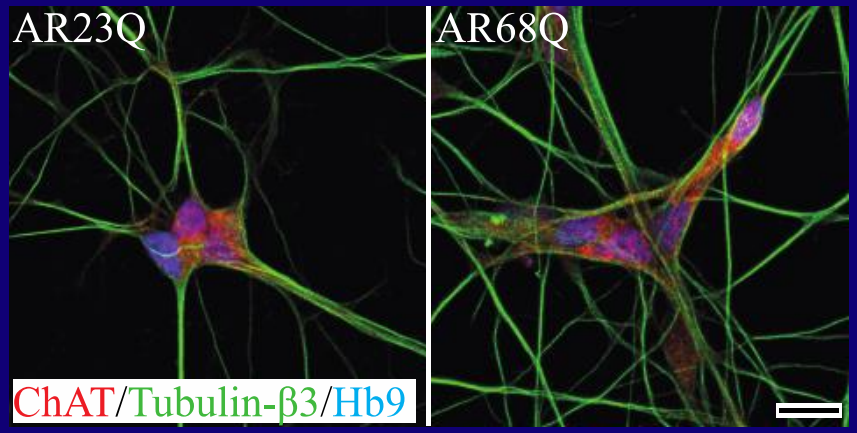
PCR amplification of predicted off-target sequences

DNA sequencing analysis of predicted off-target effects (on the top 50 predicted off-target regions, 15 are in exon or exon-intron junction regions).

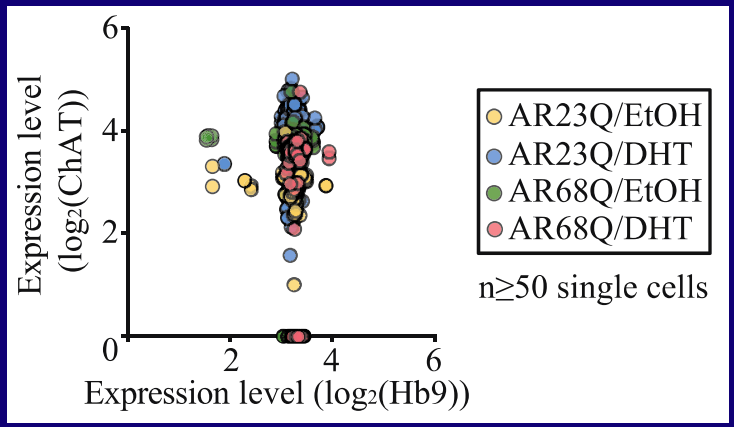
Motor neuron differentiation using a chemical-directed approach



Schematic of MN differentiation using small molecule cocktails



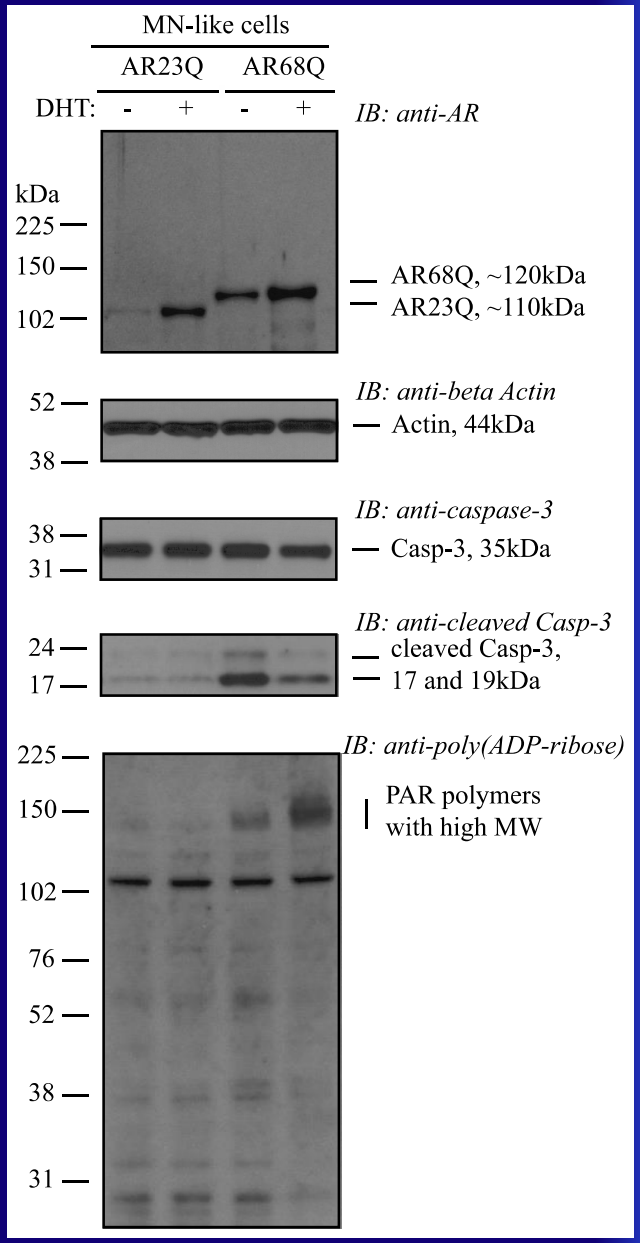
Differentiated MN-like cells express:
spinal MN markers –Hb9, ChAT (choline acetyltransferase)
neuronal marker – Tuj1 (tubulin beta-3)



Differentiation efficiency is ~90%
(Single-cell gene profiling)

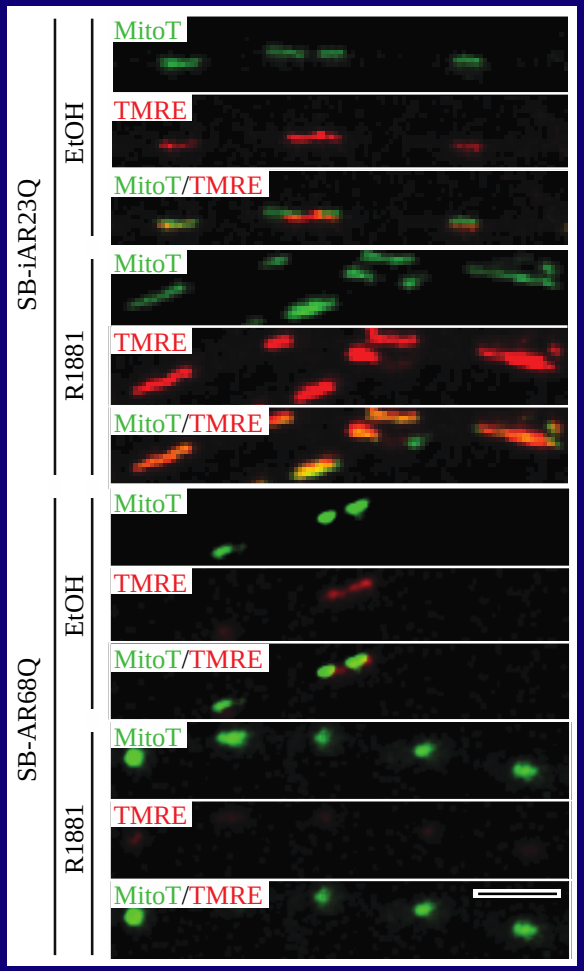
MN-like cells expressing mutant AR show cytotoxicity

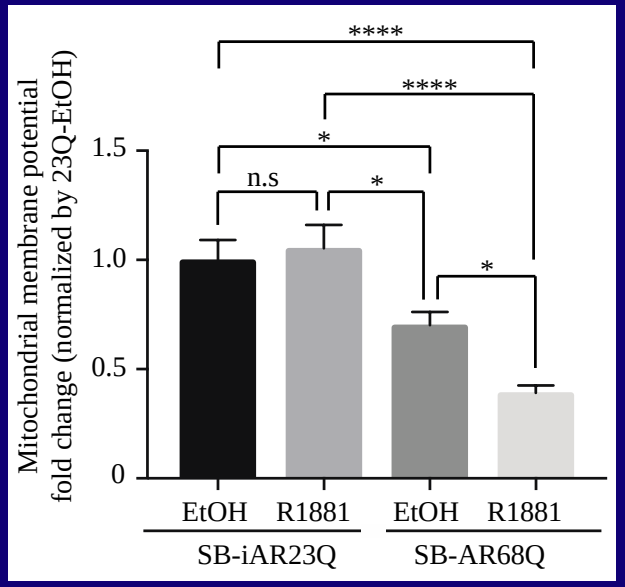
- Disease cells show elevated cleaved Caspase-3
  - Cleavage Caspase-3: products of activation of Caspase-3 in apoptotic cells
  - Increased Caspase-3 activity is a sign of apoptosis (programmed cell death)
- Disease cells show significantly increased PARylation upon ligand treatment
  - PARylation: poly-ADP-ribosylation, a post-translational modification of many proteins involved in DNA damage response, chromatic reorganization, and apoptosis.
  - Increased PARylation is an indicator of DNA damage.



MN-like cells expressing mutant AR have impaired mitochondria

-- SB-AR68Q vs. SB-iAR23Q

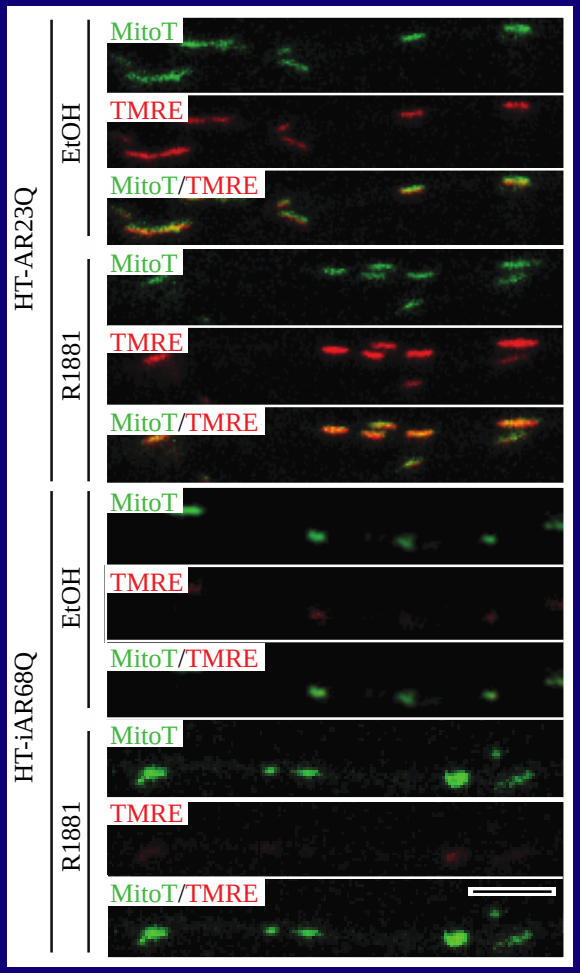


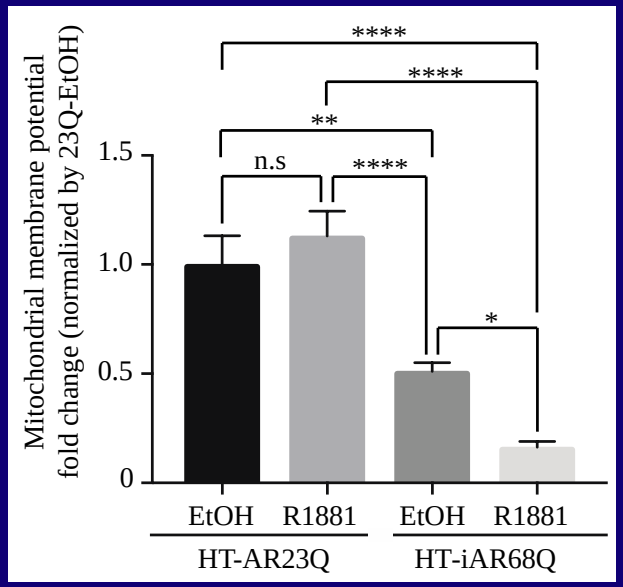


MitoT stains mitochondria;
TMRE stains mitochondrial membrane potential
Mitochondrial depolarization is an early event of cytotoxicity.

MN-like cells expressing mutant AR have impaired mitochondria

-- HT-iAR68Q vs. HT-AR23Q

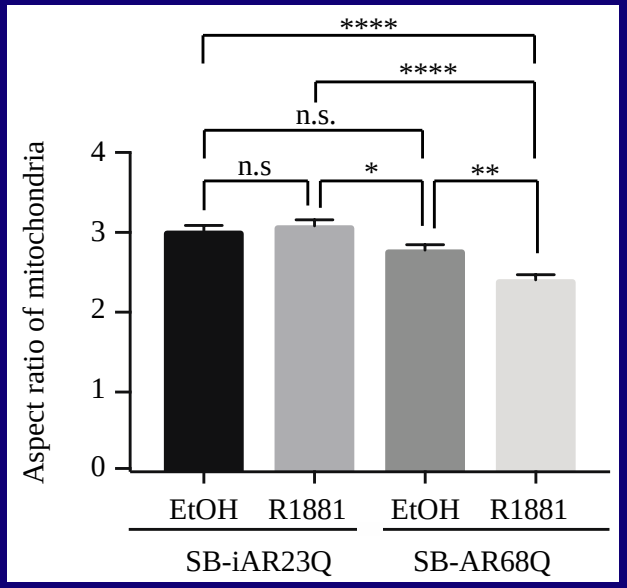


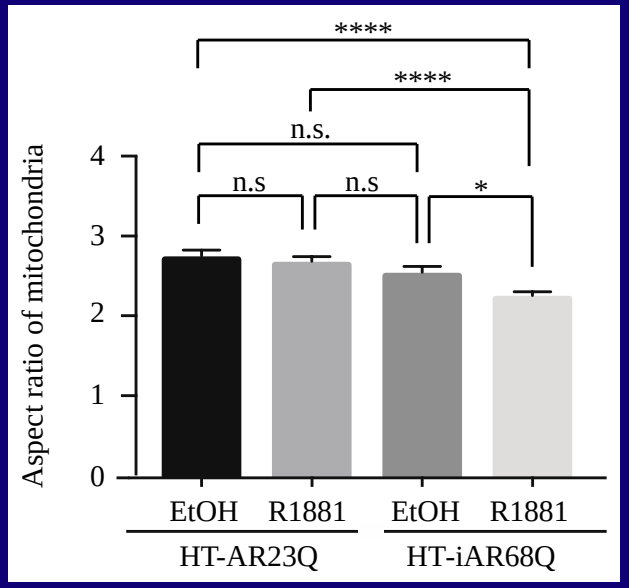


MitoT stains mitochondria;
TMRE stains mitochondrial membrane potential
Mitochondrial depolarization is an early event of cytotoxicity.

MN-like cells expressing mutant AR have impaired mitochondria

-- significantly decreased aspect ratio upon ligand treatment





Aspect ratio of mitochondria: a measure of mitochondrial shape.
Decreased aspect ratio suggests more fragmented mitochondria.

To identify new interacting proteins of mutant AR in SBMA motor neuron-like cells:
-- label-free quantitative mass spectrometry and proteomics

- *Sample preparation:* differentiated isogenic MN-like cells expressing wiltype or mutant AR as well as AR knockout will be treated with vehicle or ligand for 8 days, followed by co-immunoprecipitation (co-IP) using AR-specific antibody.
  - The AR knockout lines will serve as cell line-negative controls to clear background noise due to antibody non-specific binding in co-IP experiments.

- *Pay attention to*: candidates are in a ligand-dependent and polyQ-specific manner, or up- or down-regulated in the disease cells comparing with control cells.

- *Proteomic analysis:*
  - Gene ontology term identification: connect proteins with their encoding gene ontology terms (cellular component, molecular function, and biological process)
  - Enrichment analysis: compare abundance of the proteins
  - Pathway analysis: sort proteins into cellular signaling pathways
  - Survey of protein interactions: reconstruct MN-specific mutant AR-protein interactions

Ligand-dependent and polyQ-specific binding proteins

-- biochemical and physiology significance

- *Verification and validation:* interaction must be confirmed first through AR co-IP and reserve immunoprecipitation assays, and co-localization studies.

- *Biochemical significance*: binding interface is an important consideration for therapeutic design.
  - Determine which domain in AR protein is involved in the interaction using truncated variants.
  - Predict potential binding sites in candidate protein using sequence comparisons.
  - Evaluate the binding contribution of candidate protein to cellular localization, stability, turnover, and aggregation of the mutant AR.

- *Physiological significance:*
  - As a deleterious modifier: increases protein stability or decrease turnover of the ligand-bound mutant AR, and/or facilitate hormone binding-induced cytotoxicity.
  - As a protective modifier: promotes degradation or reduce stability of the mutant AR, and/or decrease cytotoxicity upon ligand binding.

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